Background Summary for the September 25, 2008, VRBPAC Meeting: Use of MDCK Cells for Manufacture of Live Attenuated Influenza Vaccines

Introduction

Influenza vaccines have been manufactured with embryonated hens' eggs using processes that have changed very little in the past 60 years. While egg-based manufacturing is well established and has a proven safety and efficacy record, it does have limitations. For example, certified eggs for the annual influenza vaccine have to be ordered with a lead-time of up to one year, which limits flexibility of manufacture. As such, scale-up of manufacturing in response to increased vaccine demand, to unexpected vaccine supply shortages, or to threats of pandemic influenza could be limited or delayed based on the availability of embryonated eggs. In addition, some influenza virus strains grow poorly in embryonated eggs resulting in low production yields and/or the generation of virus variants that are antigenically different from the original starting virus *(*Robertson 1990, Robertson 1993, and Schild 1983).

To address these issues, the U.S. Department of Health and Human Services has provided funding to multiple manufacturers to develop cell-culture systems for producing both seasonal and pandemic influenza vaccines. These systems could be used to produce vaccines of the same basic design as current egg-based production systems – i.e., production of whole-virus, inactivated split-virion, purified antigens, or live attenuated influenza vaccines – while at the same time eliminating the long lead times and supply-chain vulnerabilities required for egg-based production systems. Cell-culture based production systems also have the potential of offering additional benefits such as:

- Increased production uniformity – manufacturing utilizes extensively characterized cells (cell banks) instead of eggs, properties of which vary within natural limits.

- Reduced risk of introducing exogenous or endogenous adventitious agents. Cells are required to be free of contaminating agents and are grown in closed systems, in contrast to egg-based production, which is primarily an open system.

- No egg proteins – thus, cell-culture derived vaccines could be recommended for people with egg allergies.

One of the cell lines that is being pursued for influenza vaccine production is the Madin-Darby canine kidney (MDCK) cell line which was derived from an apparently healthy adult male cocker spaniel by Madin and Darby in 1958 (Madin 1958). MDCK cells are a continuous (immortal) cell line. In contrast to some more recently established continuous cell lines produced by genetic engineering, the source of the transformation of MDCK cells to a continuous cell line is

unknown. MDCK cells are attractive for manufacturing because higher yields of influenza virus are often obtained compared with other continuous cell lines such as Vero (Merten 1996, Youi 2004). MDCK cells have been reported to exhibit varying degrees of tumorigenicity, depending upon which cells are tested and the model in which they are tested.

In November 2005, the Vaccines and Related Biological Products Advisory Committee (VRBPAC) discussed the use of MDCK cells for the production of inactivated, purified subunit, trivalent influenza vaccines (TIV) from 2 manufacturers, Solvay Pharmaceuticals and Novartis Corporation. Data presented at that time indicated that these MDCK cell banks were tumorigenic. MDCK cell banks used by Solvay had moderate tumorigenic potential (i.e., 10^5 cells were required for tumor development in nude mice at the inoculation site), whereas the Novartis cell bank demonstrated an even higher degree of tumorigenicity (as few as 10 cells were sufficient for tumor formation in nude mice). There was general agreement that the MDCK cells, including the highly tumorigenic variant described by Novartis, could be used for the manufacture of inactivated influenza vaccines. Although the committee expressed some concerns about using tumorigenic cells due to the possible presence of unrecognized occult agents, there was general consensus that the inactivation and purification steps employed in the manufacture of subunit inactivated influenza vaccines were sufficient to assure product safety against such theoretical concerns.

The purpose of this meeting of the VRBPAC is to discuss the use of MDCK cells for the production of a live- attenuated influenza vaccine. This vaccine is similar in characteristics to the licensed FluMist, which is a live, trivalent vaccine for administration by intranasal spray. The influenza virus strains for this vaccine are cold-adapted (*i.e*., they replicate efficiently at 25°C, a temperature that is restrictive for replication of many wild-type influenza viruses); (b) temperature-sensitive (*i.e*., they are restricted in replication at 37°C (type B strains) or 39°C (type A strains), temperatures at which most wild-type influenza viruses grow efficiently); and (c) attenuated (they do not produce classical influenza-like illness in the ferret model of human influenza virus infection). The cumulative effects of the antigenic properties and the cold-adapted, temperature-sensitive, and attenuated phenotypes are that the attenuated vaccine viruses replicate in the nasopharynx to induce protective immunity without inducing the clinical symptoms of influenza. The MDCK cell bank used by MedImmune is significantly different from those discussed with the Committee in 2005. To limit the theoretical concerns about the tumorigenic potential of MDCK cells, MedImmune has developed a cell bank of MDCK cells that is reportedly non-tumorigenic (i.e., no progressively growing tumors developed in nude mice receiving 10^7 cells). This was accomplished by isolating subpopulations of MDCK cells with low-tumorigenic potential from the original MDCK cell line obtained from ATCC and development of a serum-free medium for stabilizing the low-tumorigenic phenotype of this new cell bank.

Defined-Risks Approach for Neoplastic Cell Substrates

To address the regulatory issues associated with the use of neoplastic cell substrates for vaccine production, the Office of Vaccines Research and Review (OVRR), in the late 1990s, developed a Defined-Risk Approach (DRA). The principal elements of DRA involve:

1. Identifying the possible risk events.
2. Estimating or determining the frequency with which the risk event might occur.
3. Estimating the frequency of the risk event per dose of vaccine.
4. Developing assays that can be used to detect the risk event
5. Developing and validating processes that can be used to mitigate the risk to an acceptable level.

The intention of this framework was: a) to examine, and wherever possible, to quantify the potential risk of "transmitting" those components of the neoplastic cell substrate that are associated with its neoplastic activity; and b) to determine whether that "transmission" might pose a risk of cancer and other neoplastic diseases to vaccine recipients. Factors that influence the risk include (1) the level of knowledge about the mechanism leading to the development of tumorigenic cells; (2) residual cell-substrate DNA size and quantity; and (3) the presence of adventitious agents, especially oncogenic viruses. This approach was presented to the Committee in 1998. The details of subsequent CBER discussions in 2000 and 2001 with the Committee regarding the DRA were described in detail in the Background Summary provided for the November 16, 2005 VRBPAC meeting. As the current meeting represents an extension of the discussions in 2005, a copy of the November 16, 2005 Background Document is appended.

As mentioned above, the Committee previously considered the use of MDCK cells for the manufacture of inactivated influenza vaccines in 2005. One goal of the meeting was for the Committee to comment on OVRR's DRA as applied to highly tumorigenic MDCK cell substrates. Again, the main safety concerns were the potential presence of adventitious agents, particularly of unrecognized oncogenic viruses, and the amount and the form of the residual DNA present per vaccine dose. OVRR's approach to addressing these issues was to develop a comprehensive testing strategy to be completed by manufacturers that included:

1. Enhanced tumorigenicity testing that was quantitative and more comprehensive than previous single-dose, short-term assays
2. Assays for oncogenic agents – lysates prepared from the cell substrate were to be inoculated into newborn mice, newborn rats, and newborn hamsters.

3. Expanded adventitious agent testing of cell banks and virus seeds by incorporation of PCR-based methods such as product-enhanced reverse transcriptase (PERT) assay for the detection of all known retroviruses, PCR assays for the detection of specific viruses, as well as broadly reactive PCR-based assays for the detection of virus families
4. Assays designed to detect the presence of latent viruses based on treatment of the cell substrate with virus inducers followed by general detection methods (e.g., PERT assay, TEM, generic PCR).
5. Quantitative assessment of clearance and/or inactivation of different viruses afforded by each step of the manufacturing process.

The Committee noted that the CBER had set high standards for evaluating risk associated with the use of highly tumorigenic cell substrates and that the manufacturers of the inactivated influenza vaccines were meeting these standards. Concern was expressed by the majority of the Committee over the difficulty of assessing possible long-term oncogenic activity associated with the components of neoplastic cell substrates. The Committee suggested that one way to evaluate this possibility would be to inject large numbers of animals with cell lysates and follow them over their lifespan. Nevertheless, Committee members were in general agreement that MDCK cells could be used for manufacture of inactivated, subunit, influenza vaccines -– but further discussions would be needed for situations where inactivation was not applicable or when purification was not as comprehensive.

The Current Meeting

The consideration of MDCK cells for the manufacture of a live attenuated influenza vaccine has prompted this meeting. Although the MDCK cell line employed by the sponsor appears to be non-tumorigenic, OVRR's regulatory approach and recommendations to sponsors for extensive testing has generally been the same as that used for highly tumorigenic MDCK cells. Testing includes the same comprehensive oncogenicity, tumorigenicity, and adventitious agent assays as described to the committee in the 2005 — the notable exception being a lack of quantitative assessment of clearance and inactivation of model viruses afforded by the manufacturing process used to produce inactivated influenza vaccines.

Characterization of the MDCK Cell Substrate Tumorigenic Phenotype

While the use of tumorigenic cell lines such as MDCK for vaccine manufacture expands the repertoire of cell substrates available for product development, concerns posed by the use of such cells include the potential for an increased in the risk of adventitious agent contamination, especially oncogenic viruses, and the potential risk associated with the DNA from such cell substrates. Consequently, quantitative tumorigenicity testing has become one aspect of the comprehensive evaluation and characterization of neoplastic cell substrates.

Such testing provides important data regarding the cell phenotype and offers the possibility of detecting unsuspected adventitious agents and oncogenic viruses as well. For example, data on aberrations in tumor formation and differences in tumor histopathology, especially in tumors that develop after prolonged latent periods, can be indicative of cell substrate contamination. Obviously, the consistency and reproducibility of such data is a critical part of the overall regulatory evaluation.

There is a limited amount of published information regarding the tumorigenic phenotype of MDCK cells. In contrast to the report on the establishment of the Madin Darby ovine kidney cells and the Madin Darby bovine kidney cells (Madin 1958), the establishment of this cell line was not published, and there are only two early reports regarding MDCK cell tumorigenicity, one describing the capacity of these cells to form tumors in chicken embryos (Leighton 1970), the other describing their lack of ability to form progressively growing tumors in either adult or newborn nude mice (Stiles 1976).

In the November 2005 VRBPAC meeting, two manufacturers, Solvay Pharmaceuticals and Novartis Corporation, presented data on the tumorigenic phenotypes of the MDCK cells used for the manufacture of their inactivated subunit influenza vaccines. Each manufacturer characterized their cell banks by: (1) developing quantitative, dose-response tumorigenicity data in nude mice; (2) examining all mice used these tumorigenicity assay by necropsy and any tumor masses that formed by histopathology; (3) inoculating both MDCK cell DNA (100 µg/animal) and MDCK cell lysate (10^7 cell equivalents/animal) from these cells into newborn hamsters, newborn rats, and newborn nude mice; and (4) evaluating the DNA from tumors that arose in these newborns for evidence of canine DNA sequences. Briefly, their data showed that as few as 10 - 1000 cells derived from the Novartis MDCK cell master cell bank were capable of forming tumors in nude mice, while cells derived from the Solvay master cell bank required as many as 100,000 cells to form tumors in nude mice. The histopathology of these tumors was consistent with that expected for MDCK cells. No tumors developed in newborn animals from the three different species employed in these assays when inoculated with MDCK cell DNA or MDCK cell lysates. The single issue that was associated with the Novartis and Solvay data was the unexpected aberration in the tumor-forming responses at high cell doses, i.e., mice inoculated with 10^7 cells failed in the assays of both Sponsors to develop tumors in 100% of those mice inoculated as would be expected. This represents the inability of these MDCK cells to form tumors in animals that received either 10^5 to 10^6 tumor-forming doses (for the Novartis cell bank, 10 cells produced tumors in 3/24 mice while 10^7 cells produced tumors in only 11/24) or approximately 100 tumor-forming doses (for the Solvay cell bank, 10^5 cells produced tumors in 6/26 mice; while 10^7 cells produced tumors in 16/30 mice). The cause of these aberrations remains to be explained.

Preliminary data obtained in recent studies in a research setting at CBER revealed a tumorigenic phenotype expressed by MDCK cells obtained from the American Type Culture Collection (ATCC). Briefly, these studies have found that the cells from three of three different lots of MDCK CCL-34 cells obtained at 2-year intervals beginning in 2004 have all expressed tumorigenic phenotypes. The tumor-forming capacity of these cell lines is proportional to the dose of cells inoculated. However, there have been unexpected findings of regressing/recurrent tumor masses in some assays, complete regression of established tumor masses in other assays, the induction of a cell-dose dependent, systemic disease in newborn nude mice, and the inability to obtain from a population of cells that express tumorigenic phenotypes, a clonal population of MDCK cells that also express a tumorigenic phenotype.

The current sponsor, MedImmune, was advised to follow a rigorous evaluation of their MDCK cell substrate similar to that described above used by the other manufacturers They have obtained data (to be presented at this VRBPAC meeting) indicating that the MDCK cells derived from their master cell bank are non-tumorigenic in both adult and newborn nude mice. In addition, they have not found any evidence for the presence of adventitious agents in their MDCK cells or of oncogenic activity associated with either the DNA or the lysates derived from them.

There are several possible explanations for the differences in tumorigenicity phenotype being observed for MDCK cells at different institutions. These explanations include: an unexplained change in the expression of a tumorigenic phenotype that developed during cell line propagation at ATCC; variations within the un-cloned MDCK cell population with the emergence of cells representing different phenotypes under different conditions of passage in cell culture; the presence of an unrecognized adventitious agent; presence of unrecognized processes in the nude mouse that alters MDCK cell tumor formation; some combination of these factors. The lack of consistency being detected in the tumorigenic forming capacities of MDCK cells by different institutions and the possible explanations for these differences that need to be considered present regulatory challenges. The fact that this substrate is being used to develop a live-virus vaccine for annual use in the general population further complicates these issues.

Regulatory Issues Associated with Residual Cell-Substrate DNA

Vaccines and other biological products manufactured in cells contain contaminating residual DNA derived from that production cell substrate, with the amount and form of this DNA depending mainly on the type of vaccine and the manufacturing process. From a regulatory perspective, residual DNA is considered a risk factor, particularly for vaccines manufactured in tumorigenic cell substrates. Because DNA has demonstrable biological activities such as oncogenicity and infectivity, OVRR does not consider that DNA is an inert

contaminant. The biological activities that DNA could transfer are oncogenic activity and an infectivity activity.

DNA oncogenicity is the capacity of DNA to transform a normal cell into one that could establish a tumor in an animal. This oncogenic event could be one that fully transforms a normal host cell into one that could establish a tumor, or it could be an "initiating" oncogenic event that alone would be insufficient to convert a normal cell to a tumor cell but predisposes the cell to becoming a tumor cell by the acquisition of additional genetic or epigenetic changes. An additional consideration is the genetics of the vaccine recipient. Certain individuals are known to be genetically predisposed to cancer, and thus the acquisition of a second oncogenic event *via* DNA could have more serious consequences for those people. DNA infectivity activity could occur if the cell-substrate DNA contains the genome of an infectious virus and if this genome, when inoculated into a human vaccine recipient, results in the establishment of an infection. Moreover, because of the possibility that a virus resulting from the infectious DNA could be amplified during a productive infection, DNA infectivity might represent an even greater risk than DNA oncogenicity.

Few data exist on either DNA oncogenicity or DNA infectivity. Recent studies in a research setting at CBER have focused on developing assays that can quantify these biological activities of DNA, and the data obtained from these studies have been used to estimate risks (Peden, Sheng, Pal, and Lewis, 2006). In addition, these quantitative assays can be used to measure the efficiency by which certain manufacturing processes (*e.g.*, chemical inactivation or nuclease digestion) can reduce the biological activity of DNA. This approach was described in the November 2005 VRBPAC meeting and is briefly updated here.

Using a highly sensitive mouse strain we have now determined that lower levels of oncogenic DNA can be detected than previously found (< 1 nanogram of a dual-oncogene-expression plasmid). In addition, we have found that a single oncogene can be sufficient to induce tumors. The latency period of tumor development by these single oncogenes is extended, presumably because tumor formation required additional cellular events to occur. These results suggest that DNA might represent more of an oncogenic risk than previously recognized, both because of the low levels of DNA that can be oncogenic and the ability of single oncogenes to initiate the tumorigenesis process.

An *in vitro* DNA infectivity assay can detect 1 pg of a retroviral genome, which is about 1000-fold more sensitive than the DNA oncogenicity assay. Because of its higher sensitivity – and our decision to make estimates of risk/safety based on a worst-case situation – the DNA infectivity assay has been used to estimate the risk from DNA and has be used to determine the amount of clearance of DNA biological activity that can be achieved using chemical or enzymatic methods. Our studies have shown that clearance levels (or safety factors) of infectivity of $>10^7$ can be achieved either by chemical inactivation or digestion down to a

mean size of about 350 base pairs when a level of DNA of 10 ng per vaccine dose is considered. With such safety factors, it appears that the risk of an infectious or oncogenic event from DNA can be reduced to acceptable levels. However, such a conclusion depends on being able to determine the proportion of DNA at and above a defined size class, and there are no validated assays for this determination. Until process validation is achieved for the product, some consideration may need to be given to determining the size distribution on a lot by lot basis.

Concluding Remarks

In the current meeting of the Vaccines and Related Biological Products Advisory Committee meeting, the committee will review the available data regarding the characterization of the MedImmune MDCK cell bank and the use of these cells for production of a live attenuated influenza vaccine. In addition, the committee will discuss the overall approach used to characterize the tumorigenic phenotype of neoplastic cell substrates, whether sufficient data are available to ensure product safety in a proposed clinical trial, and whether additional data are needed before more extensive clinical studies are undertaken.

References

Gaush CR, et al. Characterization of an established line of canine kidney cells (MDCK). Proc. Soc. Exp. Biol. Med. (1966)122: 931-935.

Leighton J et al. Secretory activity and oncogenicity of a cell line (MDCK) derived from canine kidney, Science 163: 472, 1969.

Madin SH, Darby NB. Established kidney cell lines of normal adult bovine and ovine origin. Pro Soc Exp Biol Med (1958) 98, 574-576.

Merten OW, Hannoun CJC, et al. Production of influenza virus in cell cultures for vaccine preparation. Adv. Exp. Med. Biol. 397 (1996), pp. 141–151.

Peden K, Sheng L, Pal A, and Lewis A. Biological activity of residual cell-substrate DNA, in Vaccine Cell Substrates (2004), pp. 45-53.

Robertson J, Clinical influenza virus and the embryonated hen's egg, *Rev Med Virol* 3 (1993), pp. 97–106.

Robertson J, Bootman G, Nicholson C, Major D, Robertson E and Wood J. The hemagglutinin of influenza B virus present in clinical material is a single species identical to that of mammalian cell-grown virus, *Virology* 179 (1990), pp. 35–40.

Schild GC, Oxford JS, de Jong JC and Webster RG. Evidence for host-cell selection of influenza virus antigenic variants. Nature (1983) 303,706-709.

Stiles CD, et al. Growth control of heterologous tissue culture cells in the congenitally athymic nude mouse. Cancer Research 36: 1353, 1976.

Youil R, Su Q, Toner TJ, Szymkowiak C, Kwan W-S, B. Rubin B. Comparative study of influenza virus replication in Vero and MDCK cell lines. *J of Virol Methods (2004);120, 23-31.*